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Interactions between Cyclic Nucleotide Phosphodiesterase 11 Catalytic Site and Substrates or Tadalafil and Role of a Critical Gln-869 Hydrogen Bond

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ABSTRACT

Poor understanding of the topography of cyclic nucleotide (CN) phosphodiesterase (PDE) catalytic sites compromises development of potent, selective inhibitors for therapeutic use. In the X-ray crystal structures of the catalytic domains of some PDEs, an invariant glutamine hydrogen bonds with groups at C6 and N1 or N7 on catalytic products or analogous positions of some inhibitors, inferring similar bonds with CNs (*Nature* **425:**98–102, 2003; *J Mol Biol* **337:**355–365, 2004; *Mol Cell* **15:**279–286, 2004). A site-directed mutant (Q869A) lacking this invariant Gln in cGMP-/cAMP-hydrolyzing PDE11 had unaltered catalytic activity and affinity for sildenafil; but cGMP/cAMP or tadalafil affinity was reduced ~50- or 140-fold, respectively, and calculated free energy of binding suggested one hydrogen bond for each. A cGMP analog lacking the C6 oxygen had ~80-fold weakened affinity, modifications at N^2, N7, or 2′-OH diminished affinity ~16-fold, and analogs with groups appended at N1 had only 2- to 6-fold weakened affinity. Analogs with C8 substitutions were ineffective inhibitors, suggesting that cGMP binds in the *anti* conformation. Calculated decline in free energy of binding was consistent with that for one hydrogen bond only in the analog lacking binding potential at C6. In conclusion, Gln-869 interacts strongly with cGMP/cAMP and tadalafil, but not with sildenafil; interactions with CN analogs suggest a hydrogen bond only between Gln-869 and the C6 substituent. The results define interactions between the PDE11 catalytic site and substrates/inhibitors and advance potential for inhibitor design.

There are 11 human cyclic nucleotide phosphodiesterase (PDE) families. Some selectively hydrolyze cAMP or cGMP, whereas others hydrolyze both (Beavo et al., 2006; Conti and Beavo, 2007). Important advances have been gained from X-ray crystal structures of isolated catalytic domains (C domain) of several PDEs, but contacts between catalytic site amino acids of PDE holoenzymes and cyclic nucleotides (CN) or inhibitors are not fully understood for any PDE (Huai et al., 2003; Xu et al., 2004; Blount et al., 2006; Ke and Wang, 2006; Wang et al., 2006, 2007; Zoraghi et al., 2007). The PDE11 family is derived from a single gene that produces four splice variants; all hydrolyze cAMP and cGMP, although kinetic characteristics for the variants are different (Fawcett et al., 2000; Hetman et al., 2000; Yuasa et al., 2000; Weeks et al., 2007). To date, there are no potent, specific inhibitors for the PDE11 family. Tadalafil (Cialis; Lilly-ICOS Co., Indianapolis, IN), which potently inhibits PDE5, also inhibits PDE11 albeit with significantly lower affinity (Weeks et al., 2007); sildenafil (Viagra) and vardenafil (Levitra) are weak inhibitors of PDE11. Most characteristics of the PDE11 catalytic site are unknown.

Models of CNs bound in PDE catalytic sites have been based on X-ray crystal structures of isolated C domains in complex with low-affinity 5′-nucleotide catalytic product or substrate-analog inhibitors because a cocrystal of a PDE wild-type (WT) C domain with CN is not available (Xu et al., 2004; Zhang et al., 2004). In X-ray crystal structures of several isolated C domains of PDEs and in models based on these structures, the side chain of an invariant glutamine forms two hydrogen bonds with the CN (Fig. 1); in some instances, similar bonds are formed with analogous positions

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ABBREVIATIONS: PDE, cyclic nucleotide phosphodiesterase; C domain, catalytic domain; CN, cyclic nucleotide; WT, wild type; IBMX, 3-isobutyl-1-methylxanthine; BSA, bovine serum albumin; E64, (2*S*,3*S*)-3-(*N*-((*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl] 3-methylbutyl (carbamoyl) oxirane-2-carboxylic acid); AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; PMSF, phenylmethylsulphonyl fluoride; Ni-NTA, nickel-nitriloacetic acid; Sf9, *Spodoptera frugiperda*; 8-pCPT-cGMP, 8-(4-Cl-phenylthio)-cGMP; 7-CH-cGMP, 7-deaza-cGMP; PET-cGMP, β-phenyl-1,N^2-etheno-cGMP; N^2-(6-aminohexyl)-cGMP, 2-amino-hexyl-cGMP; ΔG, Gibbs free energy of binding; $\Delta\Delta G$, binding energy in enzyme-transition state complexes; PKGIα, protein kinase Iα; PAGE, polyacrylamide gel electrophoresis.

in inhibitors containing purine-like rings (Sung et al., 2003; Zhang et al., 2004; Ke and Wang, 2006; Wang et al., 2006, 2007, 2008a,b). In X-ray crystallographic structures of PDE4D in complex structures with the 5′-AMP catalytic product, or 8-Br-5′-AMP, the invariant Gln forms hydrogen bonds involving the C6 amide and N1 or N7 (Xu et al., 2004). These reports 1) predict that two hydrogen bonds are important for high-affinity interaction of PDEs with CNs and inhibitors that are structurally similar to CNs), 2) imply that the bonds are essentially energetically equal for ligand binding, and 3) suggest that modifications at C6 and either N1 or N7 of CNs or analogous positions in purine-like inhibitors would greatly diminish affinity for PDE catalytic sites, thus limiting design of inhibitors. Despite widespread acceptance, these concepts have not been verified using PDE holoenzymes.

In PDE5, site-directed mutagenesis of the invariant Gln (Q817A) caused a major loss of affinity for cGMP substrate and four inhibitors [sildenafil, vardenafil, tadalafil, and 3-isobutyl-1-methylxanthine (IBMX)] (Zoraghi et al., 2006, 2007); X-ray crystal structures indicate that three of these (sildenafil, vardenafil, and IBMX) form two hydrogen bonds with the Gln side chain involving the positions/substituents that are analogous, respectively, to C6 and N1 in cGMP; tadalafil forms only a single hydrogen bond with Gln-817 (Sung et al., 2003; Huai et al., 2004). However, calculated loss in free energy of binding for cGMP or the four inhibitors in Q817A was consistent with that for only one hydrogen bond (Zoraghi et al., 2006, 2007). Whether this pattern was unique to PDE5 or would apply to other PDEs was debated.

To advance understanding of the PDE11 catalytic site and improve the knowledge base for use in inhibitor design, we have herein investigated the role of the invariant Gln (Gln-869) as a determinant of substrate and inhibitor affinity using mutagenesis (Q869A), cGMP analogs, and a battery of CN analogs to map features of the PDE11 catalytic site. The combined results support a critical role for a single hydrogen bond between Gln-869 and CNs, or with the inhibitor tadalafil, but no hydrogen bond with sildenafil. Affinity of PDE11 catalytic site was greatly diminished for CNs lacking a C6 substituent and modestly weakened for those with modifications at the N1, N^2, N7, or 2′-OH. Analogs with groups appended at C8 did not measurably inhibit PDE11, suggesting that cGMP binds to the catalytic site in the *anti* conformation. The results define molecular mechanisms for interaction of PDE11 with substrates and inhibitors, provide new insights for design of PDE11 inhibitors, and support the interpretation that the invariant Gln of PDE11 holoenzyme forms a single hydrogen bond with the C6 substituent in catalytic-site ligands.

Materials and Methods

Materials. The cDNA encoding human PDE11A4 (PDE11A4) was a generous gift from Drs. Kenji Omori and Jun Kotera (Tanabe-Seiyaku Co. Ltd., Saitama, Japan). cAMP, cGMP, bovine serum albumin (BSA), EDTA, penicillin, streptomycin, amphotericin B, *Crotalus atrox* (western diamondback rattlesnake) venom, guanosine, adenosine, theophylline, Triton X-100, E64, AEBSF or PMSF, leupeptin, and pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO). Complete EDTA-free protease inhibitor cocktail was obtained from Roche Diagnostics (Indianapolis, IN) and was used at the manufacturer's suggested concentration. Imidazole was from Thermo Fisher Scientific (Waltham, MA). Tritiated cAMP and cGMP were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Tadalafil was synthesized based on the procedure of Daugan et al. (2000). Sildenafil was purified from Viagra tablets as described previously (Francis et al., 2003). Nickel-nitriloacetic acid (Ni-NTA) affinity resin was obtained from QIAGEN (Valencia, CA). Baculovirus expression vector system and all vectors were obtained from Invitrogen (Carlsbad, CA) and BD Pharmingen (San Diego, CA). *Spodoptera frugiperda* (Sf9) cell media were prepared by the Vanderbilt Cell Biology Core (Nashville, TN) or purchased commercially from Orbigen (San Diego, CA). Sf9 cells were purchased from BD Pharmingen or Orbigen. T-175 tissue culture flasks were obtained from Corning Inc. (Corning, NY). Precision Plus All Blue protein standards and Coomassie Brilliant Blue *R*-250 were obtained from Bio-Rad (Hercules, CA). Human or bovine PDE5A1 was expressed and purified as described previously. 2′-*O*-Monobutyryl-cGMP, N^2-monobutyryl cGMP, 2′-deoxy-cGMP, N1-methyl-cGMP, 8-(4-Cl-phenylthio)-cGMP (8-pCPT-cGMP), cGMP, cAMP, and cIMP were purchased from Sigma-Aldrich. 2-Amino-purine riboside cyclic monophosphate, 7-deaza-cGMP (7-CH-cGMP), N^2-(6-aminohexyl)-cGMP, β-phenyl-1,N^2-etheno-cGMP (PET-cGMP), 8-bromo-cGMP, 8-(2-aminoethylthio)-cGMP, and 8-(2-aminophenylthio)-cGMP were purchased from Biolog (Bremen, Germany). N1-benzyl-cGMP was a gift from Dr. J. Miller.

Construction, Expression, and Purification of Q869A Mutant of PDE11A4. The Q869A mutant of human PDE11A4 was generated as follows: the expression plasmid containing full-length PDE11A4 (pFastBac-HTc-PDE11A4) was used as template with the forward PCR primer 5′-GAACTGCCTCGGTTGGCACTGGAGTGGATTGATAG-3′ and the reverse PCR primer 5′-CTATCAATCCACTCCAGTGCCAACCGAGGCAGTTC-3′ to generate the expression plasmid containing the Q869A mutant, which was then verified by DNA sequencing and used to generate a recombinant baculovirus as described for expression in Sf9 cells. The expression and lysis conditions for the Q869A mutant were identical to those for WT PDE11A4. The Q869A mutant was purified to essential homogeneity by Ni-NTA affinity chromatography as described previously (Weeks et al., 2007). Soluble supernatant from 2.8×10^9 cells infected with the optimal amount of Q869A virus was loaded onto a 11-ml Ni-NTA column equilibrated in buffer containing 10 mM potassium phosphate, 25 mM 2-mercaptoethanol, Roche Complete protease inhibitors, 0.07 μg/ml pepstatin A, 250 μM PMSF or AEBSF, 1 μg/ml leupeptin, 5 μM E-64, and 20 to 40 mM NaCl at a final pH of 6.8. The column was then washed with 20 ml each of 20, 25, 30, and 35 mM imidazole in the same buffer. Under the conditions tested, the majority of Q869A enzyme remained bound to the column throughout these washes. The column was then washed with 20 ml (~2 column volumes) of the buffer containing 100 mM imidazole, pH 6.8. Although this stringent wash eluted a small amount (<6% of bound activity) of Q869A mutant, it removed over 90% of contaminating proteins. The column was then washed with 20 ml of the same buffer containing 35 mM imidazole and then washed with 20 ml of the same buffer that had been adjusted to pH 8.0. After this wash, the Q869A enzyme was eluted by the following method: 1 column volume of the buffer containing 100 mM imidazole, pH 8.0, was applied to the column and allowed to sit for 30 to 60 min at 4°C, after which fractions (1 ml) were collected, and the process was repeated. Eluted fractions were assayed for PDE11 catalytic activity at 300 nM [^{3}H]cGMP (described below), and elutions were repeated until the eluted activity was negligible. Fractions containing the highest activity or purity were pooled, dialyzed, and frozen as described previously (Weeks et al., 2007).

Measurement of PDE11 Catalytic Activity. Measurement of maximal catalytic activity (V_{max}), Michaelis-Menten constant (K_m) for cAMP or cGMP, and values for IC_{50} (inhibitor concentration at 50% inhibition) of inhibitors were determined using the PDE catalytic activity assay essentially as described previously (Weeks et al., 2007). PDE11 proteins were diluted in 10 mM potassium phosphate, pH 6.8, with 25 mM β-mercaptoethanol and 1 mg/ml BSA; in general, 10 μl of diluted enzyme was added to 90 μl of PDE assay mixture (50

mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mg/ml BSA, pH 7.5, with trace [^{3}H]cGMP or [^{3}H]cAMP and unlabeled CN as indicated). Reaction mixtures were incubated for various times at 30°C and terminated by addition of 20 μl of a stop mix containing 143 mM EDTA, 60 mM theophylline, 30 mM cAMP, 30 mM cGMP, and 286 mM Tris-HCl, pH 7.5. The tubes were transferred to an ice-water bath. Snake venom nucleotidase (200 μg) was added to each tube, and reactions were incubated for 30 min at 30°C before addition of 1 ml of an ice-cold stop solution (0.1 mM adenosine, 0.1 mM guanosine, and 15 mM EDTA). Reactions were applied to 2.1-ml QAE-Sephadex columns that had been equilibrated in 20 mM ammonium formate, pH 7.5, and the flow-through was collected. Columns were washed with 2 ml of ammonium formate, and the combined flow-through eluants were combined with 10 ml of scintillation fluid and counted in a Beckman scintillation counter (Beckman Coulter, Fullerton, CA). Counts (counts per minute) were converted to picomole of CN hydrolyzed per minute per milliliter of enzyme. K_m values of human PDE11A4 isozymes for cGMP and cAMP were determined using cAMP and cGMP concentrations ranging from 0.03 to 80 μM. K_m studies for PDE11 Q869A were conducted at concentration of CN up to 500 μM and with Q869A concentration of 28 to 39 nM. Reactions were experimentally determined to be linear with respect to time (data not shown). For determination of IC_{50} values for tadalafil or sildenafil, PDE11 proteins were analyzed using cGMP as substrate at a concentration that was ~10 times below the experimentally determined K_m. This method ensured that the IC_{50} obtained approached the K_i. The apparent K_m and V_{max} values were determined by nonlinear regression analysis of data with a single-site model in Prism GraphPad Software Inc. (San Diego, CA). In all studies, less than 20% of total [^{3}H]cGMP or [^{3}H]cAMP was hydrolyzed during the reaction. The K_m value approximates a dissociation binding constant (K_D) only when the enzyme-substrate complex is in thermodynamic equilibrium. Data for IC_{50} determinations were plotted and analyzed with GraphPad Prism using a sigmoidal dose-response model. For studies involving competition of unlabeled cGMP or cGMP analogs with [^{3}H]cGMP (0.1 μM) for the catalytic site, the apparent K_m/K_i for cGMP was 0.34 μM; this indicated that the substrate concentration in these particular studies was 4-fold below the K_m; therefore, the IC_{50} values for the three analogs were converted to K_i values using the following equation: $K_i = IC_{50}/1 + [S]/K_m$ (Cheng and Prusoff, 1973).

Calculation of Free Energy of Binding. All values determined represent at least three measurements, each in either duplicate or triplicate. The value for the Gibbs free energy change (ΔG) that occurs by association of substrates or inhibitors with PDE11 is related to the equilibrium association constant for the interaction and were calculated as described from $\Delta G = -RT \ln K$, where $K = K_m$ or K_i were calculated as described above. R is the ideal gas constant (equal to 1.98×10^{-3} kcal deg^{-1} mol^{-1}), and T is the temperature at which the assay was done (303°K).

The contribution of a substituted amino acid side chain to the Gibbs free energy of binding in an enzyme-transition state complex was calculated from $\Delta\Delta G = \Delta G_{WT} - \Delta G_{MUT}$, where ΔΔG is the change in free energy of binding in enzyme transition-state complexes attributable to the substituted group. ΔG_{WT} and ΔG_{MUT} are the Gibbs free energy change for WT PDE11 and mutant (Q869A), respectively.

Assays for PDE11 Hydrolysis of 1-Methyl-cGMP. PDE11A4 was highly purified as described by Weeks et al. (2007). The enzyme was added to a typical PDE reaction mixture containing 50 mM Tris HCl, pH 7.5, 0.5 mg/ml BSA, 10 mM $MgCl_2$, and 6 μM 1-methyl-cGMP or cGMP and incubated at 30°C for 30 to 60 min. Reactions were terminated by placing samples in a boiling water bath for 7 min followed by centrifugation in a tabletop centrifuge at 6000 rpm for 10 min. Extent of hydrolysis of 1-methyl-cGMP and cGMP was determined using aliquots of the supernatants in assays to activate purified cGMP-dependent protein kinase as described below.

Quantification of CN Analog Activation of cGMP-Dependent Protein Kinase. Bovine lung cGMP-dependent protein kinase Iα (PKGIα) was purified to essential homogeneity using 8-(6-aminohexylamino)-cAMP-Sepharose, and kinase activity was determined as described previously (Francis et al., 1991). PKG protein concentration was determined by the method of Bradford (1976) using staining reagent from Bio-Rad and BSA fraction V (Sigma-Aldrich) as standard. Five microliters of PKGα (0.002 μg) with a specific activity of 10 μmol/min/mg was diluted in 10 mM potassium phosphate, pH 6.8, 1 mg/ml BSA, and added to 40 μl of a PKG reaction mixture containing 150 μM of the synthetic heptapeptide (RKRSRAE), 120 μM ATP with trace [^{32}P]ATP, 20 mM magnesium acetate, 100 μM IBMX, 0.9 μM cAMP-dependent protein kinase inhibitor (5–24), and 20 mM Tris HCL, pH 7.4, in the presence and absence of 5 μl of various concentrations of cGMP, 1-methyl cGMP, or aliquots from the incubation of cGMP or 1-methyl-cGMP and PDE11A4 described above. Samples were then incubated at 30°C for 20 to 40 min. Reactions were terminated by spotting 35 μl of the 50-μl reaction mixture onto Whatman P-81 phosphocellulose cation-exchange papers to determine the amount of ^{32}P transferred to the heptapeptide substrate. The papers were immediately dropped into 75 mM phosphoric acid, washed four times, dried, and counted.

A standard curve of activation of purified PKGIα kinase using known concentrations of 1-methyl-cGMP or cGMP ranging from 0.01 to 0.4 μM was generated as described above, and a 10 μM cGMP point was included as a positive control. The extent of breakdown of the CNs when incubated with PDE11A4 was determined by analyzing samples of the supernatants in which 1-methyl-cGMP or cGMP and PDE11A4 had been incubated together for various periods of time. PDE assays containing the typical components (described above) and 6 μM [^{3}H]cGMP were conducted alongside as an additional control.

Results

Physical and Kinetic Characterization of PDE11A4 Q869A. Q869A protein was expressed using the baculovirus system in Sf9 cells and purified to essential homogeneity as described under *Materials and Methods*. Q869A migrated on SDS-polyacrylamide gel electrophoresis (PAGE) as a ~100-kDa protein (Fig. 2), the migration of which was indistinguishable from that of WT PDE11A4 (data not shown). Affinities of Q869A for cGMP ($K_m = 50 \pm 1.5$ μM) (Fig. 3, A and B) and cAMP ($K_m = 94.6 \pm 7.33$ μM) (Fig. 3, C and D) were ~50- and 60-fold weaker than that of WT PDE11A4 ($K_m = 1.0 \pm 0.07$ and 1.6 ± 0.2 μM, respectively) (Table 1). V_{max} for Q869A was similar to that previously reported for WT enzyme (Weeks et al., 2007).

Role of Gln-869 in Affinity of PDE11 for Inhibitors. The effect of substitution of alanine for Gln-869 (Q869A) on affinity for tadalafil, a reasonably potent inhibitor of PDE11, or sildenafil (Weeks et al., 2007), a weak inhibitor of PDE11, was determined using 0.1 μM cGMP as substrate. The affinity of Q869A for tadalafil ($IC_{50} = 10 \pm 3.9$ μM) was 137-fold weaker than that of WT PDE11 ($IC_{50} = 0.073 \pm 0.003$ μM) (Fig. 4A; Table 2). In contrast, affinity of Q869A for sildenafil ($IC_{50} = 4.4 \pm 1.7$ μM) was not significantly different from that for WT PDE11 ($IC_{50} = 3.8 \pm 0.75$ μM) (Fig. 4B; Table 2).

Contribution of Gln-869 to Free Energy of Binding of cGMP, cAMP, or Inhibitors. The loss in energy involved in potential interactions of the invariant Gln in PDE11 with catalytic site ligands was calculated as described under *Materials and Methods* using the results derived above. The K_m of substrates or IC_{50} of inhibitors of WT PDE11 and Q869A was used to calculate the ΔG; these values were then used to

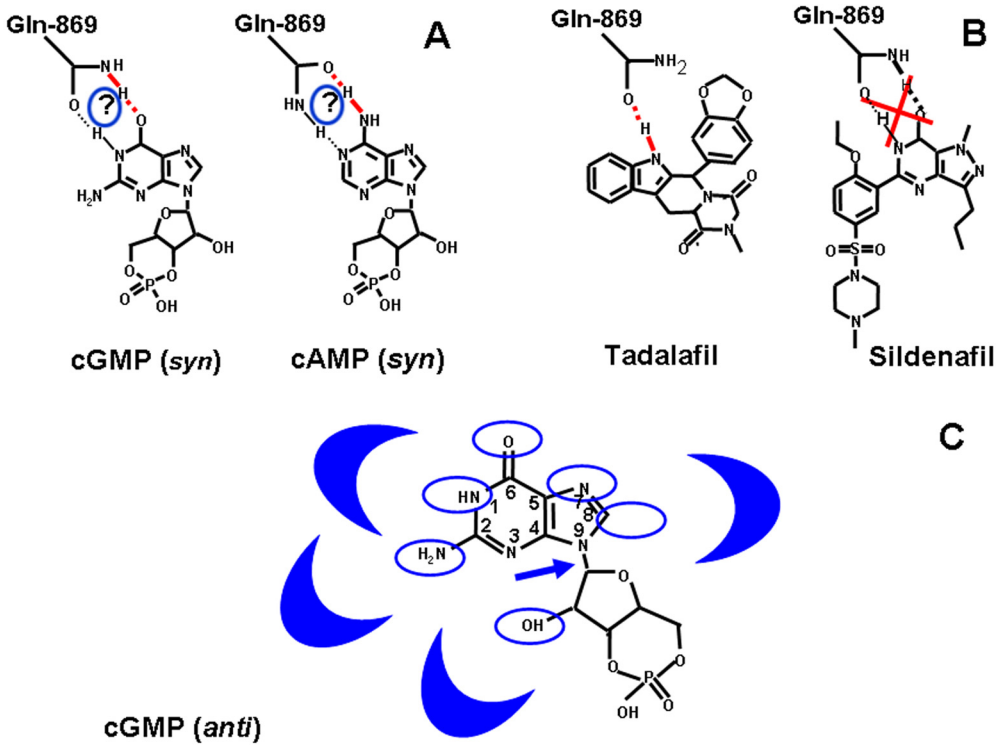

Fig. 1. Schematic depiction of interactions between the Oε and Nε of invariant glutamine (Gln-869) of PDE11 with substrates, inhibitors, and cGMP analogs. A, current theory of bidentate hydrogen bonds between an invariant Gln in PDEs and CN substrates; X-ray cocrystal structures of certain PDEs and catalytic products (5′-GMP or 5′-AMP) contain these bonds (in PDE11A4, Gln-869 would be the contact amino acid), but cocrystal structure of a PDE with a CN has not been determined. In dual-specificity PDEs such as PDE11, the Gln side chain is purportedly free to rotate to favorably orient the Oε and Nε for interaction with substituents at C6 and N1 of either cGMP or cAMP. Encircled question mark emphasizes the uncertainty of the importance of each of these bonds in PDE11 affinity for cGMP or cAMP; evidence presented in this manuscript is consistent with the critical importance of the bonds shown in red. B, depiction of proposed interaction of invariant Gln-869 of PDE11A4 with tadalafil and lack of interaction with sildenafil as supported by results reported in this article. The large red X indicates lack of evidence for interaction. C, depiction of sites of modification of cGMP analogs tested for competition with cGMP for the PDE11 catalytic site are indicated by blue ovals; blue arrow indicates ribosyl bond at N9, and large blue half-moon indicate areas evaluated for spatial constraints.

calculate the loss in free energy of binding (ΔΔG) in the Q869A protein compared with WT PDE11. The loss in free energy of binding (ΔΔG) of cGMP, cAMP, tadalafil, or sildenafil, respectively, to Q869A was calculated to be 2.4, 2.4, 3.0, and 0.1 kcal/mol. These values reflected the change in binding affinities for the respective ligands caused by loss of the Gln-869 side chain in the PDE11 catalytic site, and the loss in free energy of binding for cGMP, cAMP, and tadalafil was consistent with that for a single hydrogen bond (2.5–4 kcal/mol) (Wilkinson et al., 1983; Andrews et al., 1984).

Mapping of PDE11 Catalytic Site Interactions Using CN Analogs: Assessment of Importance of Contacts with N1, N^2, N7, C6 Oxygen, C8, or 2′-Hydroxyl of cGMP on Affinity for PDE11. CN analogs were then used in a complimentary study to assess the relative importance of two putative hydrogen bonds between the WT PDE11 Gln-869 side chain and the C6 oxygen and N1 of cGMP, to systematically define the relative importance of various structural features for high-affinity interaction of PDE11 with substrate, and to characterize the physical and chemical properties of the PDE11 catalytic site that could affect binding of potential inhibitors. A range of concentrations of either unlabeled cGMP or CN analogs was added to a typical PDE reaction mixture as described under *Materials and Methods* using 0.1 μM [^{3}H]cGMP as substrate, and potency (IC_{50}) of inhibition, or competition, by unlabeled cGMP or cGMP analogs was determined. The IC_{50} values were converted to K_i as described under *Materials and Methods*. K_i for unlabeled cGMP competition with [^{3}H]cGMP hydrolysis in this series of experiments was 0.34 ± 0.03 μM (Table 3). These studies were conducted with cGMP analogs because more of these nucleotides containing the modifications of interest are commercially available compared with cAMP analogs.

Importance of Contacts and Space at N^2. Appending an amino-hexyl at N^2 (2-amino-hexyl-cGMP) or absence of the C2-amide as in cIMP had little or no effect on affinity of PDE11 for the CN (Table 3). However, appending a butyryl group at this position (N^2-monobutyryl-cGMP) weakened affinity for the PDE11 catalytic site by ~17-fold, suggesting that despite the relative unimportance of N^2 for substrate interaction with the PDE11 catalytic site, there are spatial constraints in this region that could restrict inhibitor designs using this position. Appending a large aromatic phenyletheno group (PET-cGMP), which modifies both N1 and N^2, caused only a ~6-fold decrease in affinity for the cGMP molecule; the effects of these CN analogs with modifications of the pyrimidine ring of cGMP uniquely define features of this region of the PDE11 catalytic site.

Importance of Contacts and Space at the Ribose 2′-Hydroxyl. Deletion of the 2′-hydroxyl of cGMP (2′-deoxy-cGMP) diminished affinity only 1.6-fold, indicating that contact with this position was not particularly important in interaction with the cGMP substrate. However, appending a butyryl group at this position (2′-*O*-monobutyryl-cGMP) de-

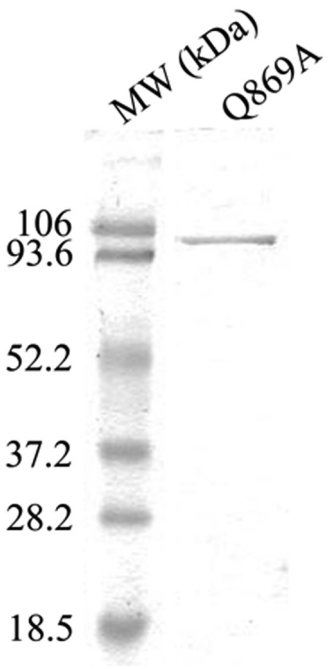

Fig. 2. SDS-PAGE of purified Q869A mutant of PDE11A4. Shown is a 10% SDS-PAGE gel stained with Coomassie Brilliant Blue containing of molecular weight (MW) standards and 2.2 μg of purified Q869A mutant of human PDE11A4. Standards (MW) are Precision Plus All Blue (Bio-Rad). The Q869A mutant was also highly pure by silver stain analysis (data not shown).

creased affinity by ~15-fold, consistent with a steric effect that could constrain future design of PDE11 inhibitors.

Importance of Contacts and Space at N1 or N7. In addition to testing the effect of PET-cGMP, which is modified at both N1 and N^2, two additional N1-substituted cGMP analogs were tested for potency in competing with cGMP. N1-benzyl-cGMP, which contains a large hydrophobic substituent at N1, was only 1.8-fold weaker (K_i = 0.61 ± 0.13 μM) (Table 3) for inhibiting breakdown of [^{3}H]cGMP than cGMP. N1-methyl-cGMP (K_i = 1.28 ± 0.13 μM) was ~4-fold less effective than unlabeled cGMP. The results provided little support for a meaningful contact of the catalytic site with the N1 of cGMP and countered the accepted interpretation that the side chain of the invariant Gln forms a strong bond with this position in the purine ring. In addition, these results revealed considerable spatial tolerance for substituents at this position and in the region extending from N1 and N^2.

Realization of the importance of contact with N7 of CNs in some PDE catalytic sites is longstanding (Couchie et al., 1983). To quantify the role of this position in PDE11, N7 was replaced with a carbon (7-deaza-cGMP). This compound had 16-fold lower affinity for the PDE11 catalytic site (K_i = 5.4 ± 2.0 μM) than did cGMP; the extent of the change in affinity with this modification was similar to that found previously with certain other PDEs (Couchie et al., 1983).

Importance of the C6 Oxygen of cGMP in Affinity for PDE11 Catalytic Site. To assess the contribution of the C6 oxygen in cGMP to high affinity for the PDE11 catalytic site, 2-amino-purine riboside cyclic monophosphate was used. As seen in Table 3, this modification produced a dramatic drop (82-fold) in affinity (K_i = 28 ± 6 μM). Deletion of the C6 oxygen caused a 5-fold greater reduction in affinity for cGMP than was observed in any of the other CN analogs; this activity emphasized the critical importance of the group appended at C6 for high-affinity interaction.

Importance of Substitutions at C8. Four analogs containing substitutions at C8 were tested and were entirely ineffective up to 20 μM for inhibition of cGMP hydrolysis (Table 3). Substitutions at C8 introduce bulk in this region, which could sterically interfere with structures in the catalytic site. In addition, the bulk of these same substitutions constrain rotation around the N9 ribosyl bond thereby favoring the *syn* conformation (Fig. 1A) of the nucleotide, suggesting that cGMP is bound in the *anti* conformation (Fig. 1C).

Change in Free Energy of Binding Due to Changes in cGMP Structure. The loss in energy of interaction between PDE11 catalytic site and various CNs due to modifications in the structure of the purine ring or groups appended to various positions of cGMP was calculated as described under *Materials and Methods*. The IC_{50} value of each of the various CN analogs compared to that of cGMP was used to calculate the ΔG, and these values were used to calculate the loss in free energy of binding (ΔΔG) due to modification of the cGMP structure (Table 3). Modifications at the 2′-hydroxyl (2′-*O*-monobutyryl-), N7 (7-deaza-), or N^2 (N^2-monobutyryl) caused loss in free energy of binding of ~1.7 kcal/mol. The greatest loss of free energy of binding (2.65 kcal/mol) was due to deletion of the C6 carbonyl oxygen and was consistent with loss of a single hydrogen bond (Wilkinson et al., 1983; Andrews et al., 1984).

Evaluation of N1-Methyl-cGMP as a Substrate for PDE11. Although N1-substituted cGMP analogs were potent competitors for cGMP at the PDE11 catalytic site, it was important to assess whether the analog was positioned in the site to allow for hydrolysis of the cyclic phosphate bond as occurs with the natural substrate, cGMP. For this reason, PDE11 hydrolysis of N1-methyl-cGMP was tested as described under *Materials and Methods*. N1-Methyl-cGMP was hydrolyzed at approximately half the rate of hydrolysis of cGMP (data not shown). The results supported the interpretation that interaction of cGMP, the natural substrate, and N1-methyl cGMP with the PDE11 catalytic site are similar.

Discussion

PDEs are prime targets for pharmacological intervention in biological processes; consequently, members of this protein superfamily have been the focus of major research efforts by medicinal chemists in academia and the pharmaceutical industry. PDE inhibitors (e.g., theophylline and dipyridamole) have been used for years in myriad therapies, but the success of potent and selective PDE5 inhibitors in treatment of erectile dysfunction [sildenafil, tadalafil, and vardenafil] and pulmonary hypertension [sildenafil (Revatio)] has renewed optimism that selective targeting of other PDEs could yield successful therapeutics in a variety of maladies. To this end, there has been a major effort to determine the X-ray crystal structures of the PDEs to 1) map the topography of the respective catalytic sites and 2) define contacts and constraints in interaction between these proteins and catalytic site ligands such as inhibitors and substrates (Wang et al., 2005, 2007; Zhang, 2006). Appreciation of the role of certain contacts and the spatial accommodations/restrictions around positions in a CN or a structurally related inhibitor in deter-

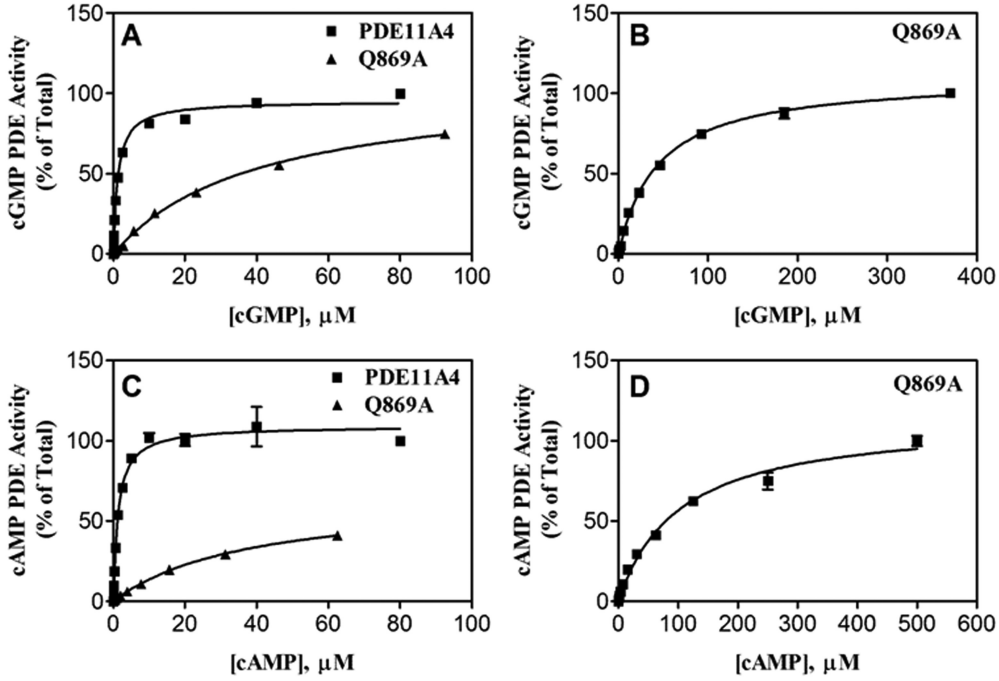

Fig. 3. Comparison of hydrolysis of cGMP and cAMP by WT PDE11 and Q869A mutant. Phosphodiesterase assays were conducted as described under *Materials and Methods*. Data are derived from three experiments performed in triplicate and analyzed by use of Prism GraphPad Software (single-site model). Error bars indicate S.E.M. Points without visible error bars indicate that the error is within the size of the symbol. Experiments were conducted at cGMP concentrations ranging from 0.03 to 380 μM (A and B) and at cAMP concentrations ranging from 0.03 to 500 μM. C and D, percentage of total on the ordinate label indicates percentage of maximal activity.

mining the affinity with which PDEs interact with ligands is therefore of utmost importance.

To date, the X-ray crystal structures of PDEs include only those of isolated C domains, and there is currently no published structure for PDE11. It has been demonstrated using solution biochemistry that many of the kinetic characteristics of an isolated C domain can be quite similar to those of the holoenzyme (Fink et al., 1999; Wang et al., 2006; Weeks et al., 2007). However, other characteristics of PDE holoenzymes can influence contacts and potency of certain inhibitors (e.g., dimerization, phosphorylation, or additional structural features) (Richter and Conti, 2004; Blount et al., 2006). Because these earlier reports demonstrated that significant structural differences can exist between a PDE holoenzyme and its isolated C domain in solution, it seems equally plausible that contacts defined from an X-ray crystal structure of an isolated C domain and certain ligands may not precisely recapitulate those that occur in the holoenzyme. Thus, for development of the most accurate insights into the biochemical properties of interaction between PDEs and ligands, it is important to compare results derived from biochemical studies of PDE11 holoenzyme in solution with insights and predictions derived from X-ray crystal structures of other PDEs (Sung et al., 2003; Zhang et al., 2004; Blount et al., 2006).

TABLE 1

Comparison of substrate affinity in WT PDE11 versus the Q869A mutant

Values are given as mean ± S.E.M. The Michaelis-Menten constant (K_m) for cAMP and cGMP and calculation of the loss of free energy of binding (ΔΔG) were determined as described under *Materials and Methods*. ΔΔG values were calculated from the K_m and not independently measured. -Fold change was calculated by dividing the Q869A value by that for WT PDE11A4. Results are derived from results from at least three experiments in which each was assayed in triplicate.

Substrate	Wild-Type K_m	Q869A K_m	-Fold Change	Calculated ΔΔG
	μM	*μM*		*kcal/mol*
cGMP	1.0 ± 0.07	50 ± 1.5	50	2.35
cAMP	1.6 ± 0.2	94.6 ± 7.33	59	2.45

In this report, we have used site-directed mutagenesis, CN analogs, and PDE inhibitors to define biochemical characteristics of the PDE11 catalytic site; these are classic approaches that have been used fruitfully to characterize the catalytic sites of many PDEs (Thomas et al., 1992; Beltman et al., 1995; Butt et al., 1995; Zoraghi et al., 2007). Herein, we demonstrate that invariant Gln (Gln-869) forms a critical

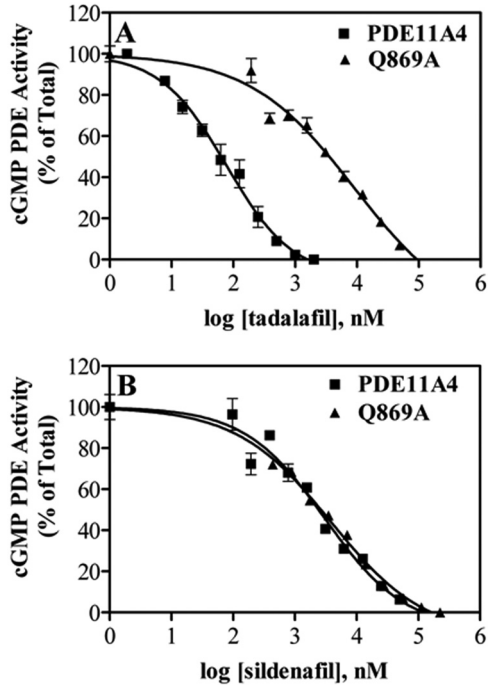

Fig. 4. Potency of tadalafil and sildenafil inhibition for PDE11 versus the Q869A mutant. The IC_{50} for tadalafil (A) and sildenafil (B) for PDE11A4 (■) and Q869A (▲) was determined as described under *Materials and Methods*. Errors bars are ±S.E.M., and points without visible error bars indicate that the error is within the size of the symbol. Data are representative of those from three separate experiments, assayed in duplicate or triplicate. Percentage of total indicates percentage of maximal activity.

TABLE 2

Comparison of potency of sildenafil and tadalafil for WT PDE11 versus the Q869A mutant

Values are given as mean ± S.E.M. The concentration at IC_{50} value for WT PDE11A4 and the Q869A mutant and calculation of the loss of free energy of binding (ΔΔG) were determined as described under *Materials and Methods*. ΔΔG values were calculated from the K_i values and not independently measured. -Fold change was calculated by dividing the Q869A value by that for the WT PDE11. Results are derived from at least three experiments in which each was assayed in triplicate. K_i for tadalafil and sildenafil for PDE11 and the Q869A mutant were calculated as described previously (Cheng and Prusoff, 1973).

Inhibitor	Wild-Type IC_{50}	Q869A IC_{50}	-Fold Change	Calculated ΔΔG
	μM	*μM*		*kcal/mol*
Tadalafil	0.073 ± 0.003 (K_i = 0.066)	10 ± 3.9 (K_i = 9.98)	137	3.01
Sildenafil	3.8 ± 0.75 (K_i = 3.46)	4.4 ± 1.7 (K_i = 4.39)	1.2	0.14

contact with CN substrates and tadalafil, which is energetically equivalent to a single hydrogen bond, that cGMP binds to the PDE11 catalytic site in the *anti* conformation, that the C6 oxygen in cGMP forms a critical contact with PDE11, that the change in free energy of interaction upon deletion of this oxygen is energetically equivalent to one hydrogen bond, and that interaction between PDE11 and N1 in CN is weak or nonexistent. Deletion of the C6 oxygen of cGMP caused a loss in affinity that is at least a five times greater than the effects of modification of other positions, and none of the latter effects equates to the energy of a single hydrogen bond (2.5–4 kcal/mol). Change in affinity due to appending groups to N1 caused 22- to 46-fold lower effects than that observed after deletion of the C6 oxygen. The results emphasize the importance of contact between PDE11 catalytic site and this oxygen and argue against a hydrogen bond with N1. Moreover, spatial tolerance for groups appended at N1 indicates that this position can be modified in efforts to improve inhibitor potency or selectivity. Based on our own work, including the results reported herein, and that of others (Thomas et al., 1992; Beltman et al., 1995; Butt et al., 1995), there seems to be no particular relationship between the selectivity of PDE catalytic sites for CN substrate(s) and the conformation (*anti* or *syn*) of the CN that is preferred: PDE1 (dual specificity)-*syn*; PDE2 (dual specificity)-*syn*; PDE3 (dual specificity)-*anti*; PDE4 (cAMP-specific)-*anti*; PD5 (cGMP-specific)-*anti*; PDE6 (cGMP-specific)-*syn*; and PDE11 (dual specificity)-*anti*.

Use of CN analogs to map CN binding sites on a variety of proteins has proved to be a highly useful tool (Corbin and Doskeland, 1983; Døskeland and Ogreid, 1984; Thomas et al., 1992; Beltman et al., 1995; Butt et al., 1995; Christensen et al., 2003), but there are intrinsic limitations to the interpretation of the results of such studies. Replacement of atoms within the rings or appending substituents that vary in size and/or chemical characteristics on the CN can uncover evidence for spatial constraints and direct binding contacts. However, these same modifications can produce more global changes in the CN chemistry including changes in the dipole moment, alteration in electronegativity of particular positions, influences on hydrogen-bonding potential at various points, and prevalence of the *syn* or *anti* conformer, thereby affecting the affinity for a particular CN binding site (Braumann et al., 1986; Beltman et al., 1995; Butt et al., 1995). Irrespective of these constraints, large changes in affinity with a single modification such as that observed in this study with the 2-amino-purine riboside cyclic monophosphate are likely to reflect the importance of a particular chemical component in the CN; this interpretation is further supported by the results of site-directed mutagenesis of Gln-869, which is thought to contact this position, as well as by X-ray crystal structures of a number of PDEs. Loss in affinity of the Q869A protein for substrates and tadalafil was specific because the structural integrity of the Q869A protein was verified by its mobility of SDS-PAGE and the fact that maximal catalytic activity and affinity for sildenafil were unchanged compared with WT PDE11A4.

These findings are of particular importance because, based on results obtained from X-ray crystal structures of isolated C domains in complex with ligands, it has become widely accepted that the invariant Gln side chain forms two critical

TABLE 3

Effect of modification of positions of cGMP on affinity of interaction with WT PDE11

Values are given as mean ± S.E.M. The concentration at IC_{50} value for WT PDE11A4 using cGMP or a CN analog as competitor was determined (see *Materials and Methods*) and converted to K_i using the equation of Cheng and Prusoff (1973). The loss of free energy of binding (ΔΔG) relative to cGMP was calculated from the K_i values as described under *Materials and Methods* and were not independently measured. -Fold change was calculated by dividing the K_i for each analog by that for cGMP. Results are derived using data from at least three experiments in which each was assayed in duplicate or triplicate.

Analog	K_i	K_i Analog/K_i cGMP	Calculated ΔΔG
	μM		*kcal/mol*
2-Amino-hexyl-cGMP	0.33 ± 0.003	0.97	−0.02
cGMP	0.34 ± 0.03	1.0	0
cIMP	0.46 ± 0.02	1.3	0.18
2′-Deoxy-cGMP	0.55 ± 0.09	1.6	0.29
N1-Benzyl-cGMP	0.61 ± 0.13	1.8	0.35
N1-Methyl-cGMP	1.28 ± 0.13	3.8	0.80
PET-cGMP	2.1 ± 0.2	6.1	1.10
2′-*O*-Monobutyryl-cGMP	5.2 ± 0.73	15.3	1.64
7-Deaza-cGMP	5.4 ± 2.0	15.9	1.66
N^2-Monobutyryl-cGMP	5.7 ± 0.56	16.8	1.69
2-Amino-purine riboside cyclic monophosphate	28 ± 6.0	82	2.65
8-Br-cGMP	>>>20	ND	ND
8-(2-Aminophenylthio)-cGMP	>>>20	ND	ND
8-(2-Aminoethylthio)-cGMP	>>>20	ND	ND
8-pCPT-cGMP	>>>20	ND	ND

ND, not determined.

hydrogen bonds with certain catalytic site ligands; (Sung et al., 2003; Huai et al., 2004; Wang et al., 2007). The combined results of the studies reported herein are consistent with the formation of a single hydrogen bond between the invariant Gln in PDE11 and CN substrates and with the interpretation that this hydrogen bond involves the C6 oxygen in cGMP. The results also suggest that the moderately potent inhibitor, tadalafil (but not the weak inhibitor, sildenafil), forms a critical single hydrogen bond with Gln-869 in the catalytic site. The results with PDE11 are in agreement with studies of interaction between WT and mutated (Q817A) PDE5 with cGMP, sildenafil, vardenafil, tadalafil, IBMX, or N1-substituted cGMP analogs, which suggested that the invariant Gln forms only one hydrogen bond with these ligands (Francis et al., 1990; Thomas et al., 1992; Zoraghi et al., 2007).

The affinities of the splice variants of PDE11 (PDE11A1-A4) for cGMP and cAMP vary by <4-fold, and affinities for tadalafil vary <5-fold (Weeks et al., 2007); the magnitude of these differences is less than that for one hydrogen bond in any of the variants (Wilkinson et al., 1983; Andrews et al., 1984). Despite the 51% amino acid sequence identity between the C domains of PDE11 and PDE5, there are significant structural differences because 1) PDE11 hydrolyzes cAMP and cGMP equally well, whereas PDE5 is highly cGMP-selective, and 2) the potent PDE5 inhibitor sildenafil is a weak inhibitor of the PDE11 splice variants (Weeks et al., 2007); the results presented herein suggest that the difference in potency of sildenafil for PDE5 and PDE11 may be attributable in large part to the lack of contact between sildenafil and Gln-869 in PDE11A4. Thus, a novel structural feature in the catalytic site of PDE11 strongly discriminates against optimal interaction with sildenafil. Whether this feature also weakens interaction of the PDE11 catalytic site with vardenafil is not known. However, the calculated difference in free energy of binding of vardenafil to PDE11, based on potency of inhibition of PDE11 (IC_{50} = 0.65 μM) compared to potency of inhibition of PDE5 (IC_{50} = 0.1–0.4 nM), is sufficient for at least one hydrogen bond (Wilkinson et al., 1983; Andrews et al., 1984; Blount et al., 2004; Weeks et al., 2007).

The results emphasize the importance of the invariant Gln in PDE11 in determining affinity for substrates as well as for inhibitors, and they quantify and define the contribution of the invariant Gln for interaction with catalytic site ligands. Spatial tolerance of the PDE11 catalytic site for a large hydrophobic group appended to N1 of cGMP suggests that PDE11 inhibitors containing a substituent in the analogous position should be explored. In conclusion, the findings from solution studies reported here reveal several novel features of the PDE11 catalytic site and provide insight into the future development of selective inhibitors targeting this poorly understood PDE family.

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